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HeLa Cell-Adherent Enteropathogenic *Escherichia coli* in Children under 1 Year of Age in Thailand

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Enteropathogenic *Escherichia coli* (EPEC) was isolated from 11% of 148 Hmong children under 1 year old with diarrhea at a refugee camp in northern Thailand. Of 16 children with EPEC-associated diarrhea, 11 were infected with EPEC that adhered to HeLa cells in a diffuse pattern, 3 were infected with EPEC that adhered to HeLa cells in a localized adherence (LA) pattern, and 2 were infected with EPEC that were nonadherent. In Bangkok, EPEC was isolated from 6% of 64 children under 1 year old with diarrhea and 7% of 56 children of the same age without diarrhea. Of four children with diarrhea, two were infected with EPEC with an LA pattern, and two were infected with nonadherent EPEC. Of four children without diarrhea, one was infected with EPEC with an LA pattern, one was infected with EPEC that adhered in a diffuse pattern, and two were infected with nonadherent EPEC. The 21 EPEC isolates with an LA pattern hybridized with the EPEC adherence factor DNA probe. EPEC was the only enteric pathogen identified in 16 (80%) of 20 children with EPEC-associated diarrhea. EPEC was as frequently isolated from children under 1 year old as were other bacterial enteric pathogens. The problem of identifying EPEC with pools of polyvalent antisera are described, and the need to identify additional enteropathogenic determinants of EPEC is discussed.

Enteropathogenic *Escherichia coli* (EPEC) has been defined as "diarrheagenic *E. coli* belonging to serogroups epidemiologically incriminated as pathogens, but whose pathogenic mechanisms have not been proven to be related either to heat-labile enterotoxins (LT) or heat-stable enterotoxins (ST) or to *Shigella*-like invasiveness" (8).

In studies of infants and animals with EPEC diarrhea, bacterial adherence to the small intestinal mucosa was found to be important for the induction of diarrhea (5, 18, 24, 28, 29, 37). Cravioto et al. reported that 80% of EPEC adhered to HEp-2 cells in tissue culture monolayers and HEp-2 adherence was significantly more common among EPEC than among enterotoxigenic *E. coli* or normal flora serotypes (6). Baldini et al. demonstrated that HEp-2 adherence by EPEC E2348 (serotype O127:H6) was encoded on a 60-megadalton plasmid (pMAR-2) (1, 2). The presence of this plasmid correlated with the ability of E2348 to cause diarrhea in adult volunteers (16).

The genes coding for localized HEp-2 (or HeLa cell) adherence have been cloned and used as a specific probe to identify EPEC adhesin factor (EAF) (21). Scaletsky et al. reported that *E. coli* can also adhere to HeLa cells in a diffuse adherence (DA) pattern in which bacteria cover HeLa cells uniformly (32). EPEC strain serogroups O55, O86, O111, O119, O125, O128, and O142 usually adhere in a localized adherence (LA) pattern. Serogroups, O55, O111, O127, O128, and O142, referred to as class I EPEC by Nataro et al., are considered to be the most important causes of epidemic and endemic EPEC-associated diarrhea (20). In contrast, class II EPEC, consisting of serogroups O44, O86, and O114, is rarely associated with outbreaks and is a less significant cause of sporadic diarrhea. Class II EPEC seldom adheres to tissue culture cells in an LA pattern but was isolated more often from children with diarrhea than from controls without gastroenteritis in Peru, suggesting that class II EPEC causes diarrhea by other mechanisms. The enteropathogenicity of an EAF-negative class II EPEC (O114:H2) was demonstrated in challenge studies with adult volunteers (16).

The prevalence of EPEC infections in Thailand was determined in children less than 1 year old with and without diarrhea. EPEC isolates were examined for LT and ST production, enteroinvasiveness, mannose-resistant adherence to HeLa cells, and hybridization with the EAF probe.

MATERIALS AND METHODS

Fecal specimens were collected from two groups of children with diarrhea. One group consisted of Hmong children under 1 year old with diarrhea at a refugee camp in northern Thailand in April and May 1985. Children without diarrhea were not studied since the primary object of this study was to assist physicians who did not have laboratory facilities in treating endemic diarrheal disease in young children. The other group consisted of Thai children under 1 year old with diarrhea and controls of the same age without diarrhea in the preceding 2 weeks who were seen at the outpatient clinic at Children's Hospital in Bangkok between January and June 1985. Diarrhea was defined as three or more loose stools in 24 h accompanied by fever, vomiting, or abdominal pain. Stools were cultured for *E. coli*, *Salmonella* sp., *Shigella* sp., *Campylobacter* sp., *Aeromonas* sp., *Plesiomonas* sp., and vibrios as previously described (34). Rotavirus was identified with a monoclonal immunoassay (13). Specimens were examined for *Cryptosporidium* sp. by a modified direct smear method (4).

Ten *E. coli* strains isolated on MacConkey agar were tested for LT and ST production (7, 30). In addition, 10 lactose-fermenting and any non-lactose-fermenting *E. coli* strains isolated on MacConkey medium were examined for hybridization with a radiolabeled DNA probe that identifies

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TABLE 1. EPEC serogroups isolated from children under 1 year old with diarrhea and from healthy controls

Adherence pattern (serotype) of[a]:	
Children with diarrhea	Controls
Children's Hospital	
NA[a] (O55:H−)	LA (O86:H34)
LA (O86:H34)	NA (O86:H42)
LA (O127:H6)	DA (O127:H6)
NA (O127:H19)	NA (O127:H48)
Refugee camp	
DA (O44:H18)	ND
DA (O86:H−)	ND
DA (O86:H27)	ND
DA (O86:H27)	ND
DA (O86:H27)	ND
DA (O86:H27)	ND
DA (O86:H27)	ND
DA (O111:H21)	ND
DA (O125:HR)	ND
NA (O125:H21)	ND
LA (O127:H−)	ND
LA (O127:H−)	ND
LA (O127:H−)	ND
NA (O127:H13)	ND
DA (O128:H7)	ND
DA (O128:H45)	ND

[a] NA, Nonadherent to HeLa cells; ND, not done.

enteroinvasive *E. coli* (34). Colonies that hybridized with this probe were tested in the Sereny test (33).

To identify EPEC, five *E. coli* isolates from each child were tested for slide agglutination in three pools of polyvalent OK EPEC antisera provided by the National Health Services, Wellington, New Zealand. Live organisms agglutinating in one of three pools of four antisera were then agglutinated with monovalent OK antisera.

Definitive O:H serotyping was performed by tube agglutination of unboiled and boiled bacteria with O and H antisera (3). Definitive serotyping revealed that only 20% of isolates preliminarily identified as EPEC were actually of EPEC serogroups. All strains were maintained on nutrient agar slants at 4°C.

Adherence to HeLa cells. EPEC strains were tested for mannose-resistant adherence to HeLa cells with a modification of a method described by Scaletsky et al. (32). HeLa cells (CCL6) were grown in Eagle minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 100 U of penicillin per ml–100 μg of streptomycin per ml–2 mM L-glutamine–10% fetal bovine serum at 37°C in 5% CO_2. A 3-ml volume of 10^5 cells per ml in growth medium was placed in 24-well 16-mm (diameter) tissue culture dishes (Costar, Cambridge, Mass.) with glass cover slips and incubated at 37°C in 5% CO_2 for 24 h. Cells were then washed three times in Hanks balanced salt solution and incubated with 1 ml of Eagle minimum essential medium with 0.5% D-mannose. A 25-μl volume of *E. coli* grown in 1 ml of tryptic soy broth at 37°C for 16 h was added to duplicate wells, mixed by rotation of the tissue culture plate five times on a flat surface, and incubated for 3 h at 37°C in 5% CO_2. The cells were then washed three times in sterile phosphate-buffered saline, pH 7.4, fixed with methanol, stained with 10% Giemsa stain, and examined microscopically under oil immersion. Adherence of *E. coli* to HeLa cells was determined by the method of Scaletsky et al. (32).

Colony hybridization. The EAF probe was prepared as previously described (21). pMAR22 was digested with *Bam*HI and *Sal*I (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), and the digestion fragments were separated by polyacrylamide gel electrophoresis. The 1-kilobase *Bam*HI-*Sal*I fragment was removed by electroelution and successively extracted with phenol, chloroform, and ether. After ethanol precipitation, the purified DNA fragment was labeled in vitro with [α-^{32}P]dCTP (New England Nuclear Corp., Boston, Mass.) by nick translation (17). *E. coli* isolates were examined for colony hybridization under stringent conditions with the EAF probe as described by Moseley et al. (19).

RESULTS

EPEC was isolated from 16 (11%) of 148 children under 1 year old with diarrhea at the refugee camp. Of these 16 children, 11 were infected with EPEC that adhered to HeLa cells in a DA pattern, 3 were infected with EPEC with an LA pattern, and 2 were infected with EPEC that were nonadherent (Table 1).

In Bangkok, EPEC was isolated from 4 (6%) of 64 children under 1 year old with diarrhea and 4 (7%) of 56 children of the same age without diarrhea. Of four children with diarrhea, two were infected with LA EPEC and two were infected with nonadherent isolates. One of the children without diarrhea was infected with LA *E. coli*, one was infected with DA *E. coli*, and two were infected with nonadherent *E. coli* (Table 1). The isolates rates of other enteric pathogens are shown in Table 2. None of these EPEC isolates were enterotoxigenic or enteroinvasive.

The 21 EPEC isolates with an LA pattern hybridized with the EAF probe. None of the 28 isolates with a DA pattern or the 19 nonadherent isolates hybridized with the EAF probe. The serotypes and patterns of adherence to HeLa cells of these EPEC isolates are summarized in Table 3. EPEC was the only enteric pathogen identified in 16 (80%) of 20 children with EPEC-associated diarrhea.

DISCUSSION

In the early 1970s, classic serotypes of EPEC were found to be neither enterotoxigenic nor enteroinvasive (10–12). These observations led many to question the pathogenicity of these isolates (9). Since 1977, EPEC has been recognized as a group of diarrheagenic *E. coli* distinct from enterotoxigenic and enteroinvasive *E. coli* (8). Recent reports suggest

TABLE 2. Percentage of enteric pathogens in children less than 1 year old with diarrhea in a refugee camp and in Bangkok

Pathogen	% of children infected		
	Refugee camp (n = 148)	Diarrhea (n = 64)	Controls (n = 56)
Rotavirus	21	19	2[a]
EPEC	11	6	7
ETEC[b]	9	8	2
Campylobacter sp.	5	6	5
Cryptosporidium sp.	5	2	0
Aeromonas sp.	3	6	7
Shigella sp.	0.6	5	2
Salmonella sp.	0.6	11	14
Non-O1 *Vibrio cholerae*	0.6	3	0

[a] $P < 0.005$ as determined by Fisher exact test. None of the bacterial enteric pathogens were isolated more frequently from children with diarrhea than from controls wthout diarrhea.

[b] ETEC, Enterotoxigenic *E. coli*.

that EPEC remains an important cause of sporadic infant diarrhea in less developed areas and is still a cause of outbreaks in developed countries (8). In this study, EPEC was frequently isolated from children less than 1 year old with diarrhea.

Studies of EPEC-associated diarrhea have been hampered by lack of a simple, sensitive, and reliable test to identify EPEC. Identification by agglutination with antisera is difficultto perform correctly and requires specific antisera and technical expertise. Furthermore, not all members of a given serogroup or serotype are indeed pathogenic (8, 14, 26, 31, 36). Nataro et al. found that only 50% of *E. coli* isolates that agglutinated in pools of polyvalent OK EPEC antisera in Peru were confirmed as EPEC serogroups (20). In the present study, only 20% of isolates that agglutinated in pools of OK antisera were confirmed as EPEC serogroups.

Considerable progress has been made in defining the enteropathogenic mechanisms of EPEC (8). Mannose-resistance adherence to HEp-2 cells was found to be associated with 80% of classic EPEC strains but distinctly less common in normal flora or other diarrheagenic *E. coli* strains (6). Scaletsky et al. demonstrated that EPEC adhered to HeLa cells in two distinct patterns, LA and DA (32). Nataro et al. developed a DNA probe to detect sequences coding for an EAF that enables bacteria to adhere in localized microcolonies on HEp-2 cells (21). This was a significant advance since it offered a convenient method to study the epidemiology of EAF-positive EPEC infections. In Peru, EAF-positive EPEC serogroups O55, O119, O111, O127, and O142 were significantly more often associated with diarrhea than were normal flora serogroups (20).

In Thailand, confirmed EPEC serogroups were isolated from 11% of Hmong children with diarrhea in northern Thailand and 6% of Thai children with diarrhea in Bangkok. EPEC was isolated from 7% of controls of the same age without diarrhea in Bangkok. The most prevalent EPEC serotypes isolated were O86:H27, 94% of which adhered to HeLa cells in a DA pattern, and O127:H−, 100% of which were EAF positive. Symptomatic EPEC infections appear to be less common where breast feeding continues throughout the first year of life than in areas where bottle feeding has replaced breast feeding (8). In Bangkok, EPEC was isolated as often from children with diarrhea as from age-matched controls without diarrhea. In developing countries where the prevalence of enteric pathogens is high, children either acquire immunity to enteric pathogens passively from breast milk or develop immunity from constant reinfections. Asymptomatic infections with enteric pathogens are common in developed countries (15, 23, 25). EPEC and other bacterial pathogens were isolated as often from asymptomatic controls as from children with diarrhea in Bangkok (Table 2).

While 30% of cases of diarrhea in young infants in Brazil and South Africa have been attributed to EPEC, this organism was isolated from only 6 to 11% of young children with diarrhea in Thailand (27, 35). In contrast to Peru, where EPEC serogroup O86 was EAF negative and only one of five EPEC serogroup O127 isolates was EAF positive, 70% of EPEC serogroup O127 and 9% of serogroup O86 isolates in Thailand were EAF positive (20). This suggests that the serogroups of EPEC that are EAF positive vary geographically.

As mentioned by Scaletsky et al., EAF-positive EPEC is serotype specific (31). Of 21 EPEC serogroup O86 isolates, which included four different H types, only O86:H34 was EAF positive, and of 27 EPEC serogroup O127 isolates, which included five H types, only O127:H− and O127:H6 were EAF positive. DA EPEC was also serotype specific (Table 3). Screening for EPEC serogroups is, therefore, too nonspecific to identify EAF-positive clones and probably other serotypes of EPEC that produce diarrhea by other presently undefined pathogenic mechanisms (14, 26, 31, 36).

TABLE 3. Enteroadherence of EPEC isolated from children in Thailand

Serotype	No. of isolates with the following adherence pattern:		
	DA	LA[a]	None
O44:H18	1	0	2
O55:H−	0	0	1
O86:H27	16	0	1
O86:H34	0	2	0
O86:H42	0	0	1
O86:H?	1	0	0
O111:H21	1	0	0
O125:H21	0	0	2
O125:HR	3	0	1
O127:H−	0	14	0
O127:H6	0	5	0
O127:H13	0	0	3
O127:H19	0	0	3
O127:H48	0	0	2
O128:H7	2	0	2
O128:H45	4	0	1

[a] All 21 EPEC isolates that adhered to HeLa cells in an LA pattern hybridized with the EAF probe.

A diagnostic test such as the EAF DNA probe that identifies a proven virulence factor may enable investigators to redefine the definition of EPEC. The facts that only 24% of EPEC strains isolated in Thailand and 32% of EPEC strains isolated in Peru were EAF positive and that an EAF-negative EPEC serotype O114:H2 strain induced diarrhea in adult volunteers suggest that additional virulence determinants of EPEC need to be determined (16, 20). Shiga toxin production by EPEC is one possible virulence determinant that needs to be evaluated in case control studies in children (9). Until additional enteropathogenic determinants are identified in EPEC, investigators must continue to rely on serotyping. There are unfortunately few laboratories that can determine O and H serotypes. Screening for EPEC serogroups with polyvalent antisera is today the standard method of studying EPEC, but these results will be misleading unless O and H serotypes are determined.

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